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Aberrant Hypermethylation of *APC* Tumor Supressor Gene in Acute Leukemia Patients

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ABSTRACT

Wingless Type (WNT) signaling pathway is an evolutionarily conserved pathway that is crucial for the cell fate determination, survival and expansion of lymphocyte progenitors. It has been demonstrated that deregulated WNT signaling is one of the participating mechanism underlying lymphoid leukemogenesis. Inactivating mutations and methylation in Adenomatous Polyposis Coli (*APC*) gene, a negative regulator of WNT pathway, can cause ligand independent WNT pathway simulation. In this study, promoter methylation and expression of the *APC* gene is evaluated in childhood lymphoid and myeloid acute leukemia patients (n= 118) and representative cell lines by using methylation specific PCR (MS-PCR) and real time quantitative PCR (QRT-PCR). APC gene promoter found hypermethylated in the 56% of childhood acute leukemia patients [49.2% of B-cell acute lymphoblastic leukemia (B-ALL), 62.5% of T-cell acute lymphoblastic leukemia (T-ALL) and 64.1% of Acute myeloid leukemia (AML)]. To evaluate the reflection of promoter methylation, *APC* mRNA levels were examined and found that all acute lymphoblastic leukemia subgroups have statistically lower *APC* expression levels compared to controls. Although there was no association with clinical parameters, promoter hypermethylation of *APC* gene seems to be a common epigenetic event in acute leukemia and leading to differential expression levels among different acute leukemia phenotypes.

Keywords: *APC*, methylation, Expression, Acute leukemia, WNT signaling

ÖZET

Akut Lösemi Hastalarında *APC* Tümör Baskılayıcı Geninin Artmış Hipermetilasyonu

WNT sinyal ileti yolağı evrimsel olarak korunmuş, lenfosit progenitor hücrelerin büyümesi ve farklılaşmasında görevli önemli bir yolaktır. Son yıllarda yapılan çalışmalar WNT sinyal ileti yolağının hematopoetik malignansilerde de önemli bir yeri olduğuna işaret etmektedir. WNT sinyal ileti yolağında görev alan *APC* geni, B-katenini degrede eden degredasyon kompleksi elemanlarından birisi olması nedeniyle yolağın negatif regülasyonunda anahtar role sahiptir. Gende meydana gelen inaktive edici mutasyonlar veya metillenme, WNT aktivasyonuna sebep olmaktadır. Bu çalışmada *APC* geni ekspresyonu ve metilasyonu çocukluk çağı akut lenfoblastik ve myeloblastik lösemi hastalarında (n= 118) metilasyon spesifik PZR ve eş zamanlı kantitatif PZR ile incelenmiştir. APC geni promotör bölgesi hastaların %56'sında (%49.2 B-ALL, %62.5 T-ALL %64.1 AML hastalarında) hipermetile olarak tespit edilmiştir. Metilasyonun, gen ekspresyonuna olan etkisini değerlendirmek için APC mRNA seviyeleri incelendiğinde tüm akut lenfoblastik lösemi hastalarında anlatımın kontrollere göre istatistiksel olarak azaldığı görülmüştür. *APC* anlatımı ile klinik parametreler arasında bir korelasyon gözlememekle birlikte, *APC* promotör metilasyonunun farklı ALL fenotipleri arasında farklı gen anlatım düzeylerine neden olduğu gözlenmiştir.

Anahtar Kelimeler: *APC*, metilasyon, Ekspresyon, Akut lösemiler, WNT sinyal ileti yolağı

INTRODUCTION

Wingless Type (WNT) pathway is one of the evolutionarily conserved signal transduction pathways that control several stages of development, including proliferation, cell fate, polarity and migration of cells. WNT signals are important for the survival and expansion of lymphocyte progenitors, it has been suggested that deregulated WNT signaling could be one mechanism underlying leukemogenesis.[1,2]

Most of the studies reported that up-regulation of B-CATENIN causes the stimulation of WNT pathway and overexpressed WNT proteins contribute to the pathogenesis of hematologic malignancies like acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and T cell acute lymphoblastic leukemia.[3-5] *APC* gene is a multi-domain protein and inactivating mutations or methylations lead to deregulated WNT signaling which has been firstly implicated in colorectal tumor evolution.[6,7] *APC* gene is the negative regulator of the WNT pathway, has been methylated in solid tumors especially colon prostate, breast cancers, multiple myeloma and acute leukemia.[8-12]

In our previous study we examined *APC* gene mutations in T cell and B cell ALL patients and showed that *APC* gene mutations are not a common mechanism for acute leukemia.[13] Methylation in the promoter region is a frequent, acquired epigenetic event involved in the pathogenesis of different type of human malignancies including leukemia. The purpose of this study was to determine the role of promoter hypermethylation of the *APC* gene in different acute leukemia subgroups and its association with clinic representation.

MATERIALS AND METHODS

Patients and Controls

A total of 118 acute leukemia patients (B-ALL n= 63, T-ALL n= 16, total AML n= 39) who were diagnosed at Istanbul and Cerrahpasa Medical Faculties of Istanbul University were included in this study. In our cohort, 71 patients were childhood ALL and eight patients were adult ALL. Median WBC was 42300 mcL. Four patients were positive for t(12;21), three patients were positive for t(4;11) and one was positive for t(9;22)-p190 translocation. In the AML cohort, 21 patients were pediatric and 18 were adult. Median WBC was 100.000 mcL. Seven patients were positive for t(15;17) translocation. The samples were taken at the time of diagnosis and the patients were treated according to BFM or GMALL protocols. Bone marrow samples were obtained at the time of diagnosis. The clinical features of acute leukemia patients are summarized in Table 1. B-ALL (FLEB14-4) and T-ALL (MOLT4, JURKAT and TALL-1) cell lines were also included to the study. Healthy bone

Table 1. Clinical features of the patients

	ALL (n= 79)	AML (n= 39)
Age		
Pediatric	71	21
Median (Min-Max)	12 (16 days- 18 years)	5 (1-15 years)
Adult	8	18
Median (Min-Max)	30.5 (21-72 years)	37 (20-71 years)
Sex		
Male	50	23
Female	29	15
Median WBC	42.300	100.000
(Min-Max)	1060-600.000	3700-50.000
Translocation		
t(12;21) positive	4	0
t(4;11) positive	3	0
t(9;22)-p190 positive	1	0
t(15;17)	0	7

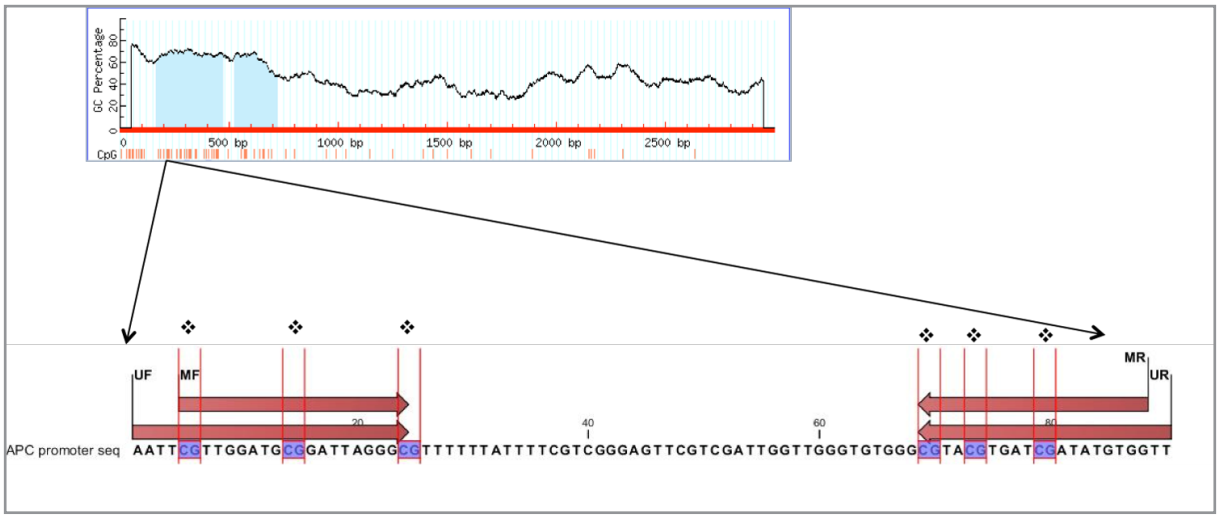

Figure 1. CpG island range (blue region) of APC gene as determined by Methprimer and methylation specific PCR primer sequences. Red fonts represent primer sequences, ❖: represents CpG islands, MF: Methylated PCR Primer Forward, MR: Methylated PCR Primer Reverse, UF: Unmethylated PCR Primer Forward, UR: Unmethylated PCR Primer Reverse

marrow (n= 10) and total thymocytes sorted from healthy thymus tissue samples (n= 7) were used as control. The ethical committee of Istanbul Medical Faculty, Istanbul University approved this study and informed consents were obtained from all patients and healthy controls (reference number and date: 2008/305 and 20.02.2008).

RNA Isolation and cDNA Synthesis

Bone marrow samples were stored at -800C after homogenization in the RTL buffer (Qiagen, GmbH, Germany). Total RNA isolated by Qiagen RNeasy Protect Kit (Qiagen, GmbH, Germany). RNA samples were treated using DNAse (1U/μg) to avoid possible DNA contamination resulting from isolation. RNA quality and quantity was checked with Nanodrop 1000 (Thermo Fisher Scientific, Germany) and cDNA was synthesized by random hexamers and MMLV reverse transcriptase (MBI Fermentase, Lithuia), from 1 μg of total RNA according to manufacturer protocol (Roche Diagnostics, Mannheim, Germany).

Bisulfite Treatment and Methylation Specific Polymerase Chain Reaction (MS-PCR)

Genomic DNA was extracted from patients' bone marrow cells by using Qiagen DNA isolation kit (Qiagen, GmbH, Germany). DNA quality and quantity was measured before NaBiS (sodium bisulphate) treatment that was described by Frommer et. al.[14] NaBiS treated DNA was purified by the Gene Clean III Kit (Q-Bio-gene) according to the manufacturer's instructions. After the bisulfite treatment, methylation specific polymerase chain reaction (MS-PCR) was used to amplify the promoter region of the APC gene in all samples.

MethPrimer (http://www.urogene.org/methprimer/index1.html) program is used to determine the CpG island range of *APC* gene and to design the MS-PCR primers; APCMF:5'CGTTGGATGCGGATTAGGGC,3',APCMR:5'CCTCATATCGATCACGTACG3',MSPCRUF:5'AATTGGTTGGATGTGGATTAGGGT3',MSPCRUR:5'AACCTCATATCAATCACATACA3' (Figure 1). A pooled DNA sample, obtained from 5 individuals, was treated with SssI methylase (New England Biolabs, United Kingdom) enzyme to obtain in vitro methylated (IVM) positive control in each run. MS-PCR results were evaluated on 3% agarose gel electrophoresis.

Analysis of Gene Expression by Real Time Quantitative RT-PCR (QRT-PCR)

Quantitative PCR (QRT-PCR) was carried out on the Light Cycler Instrument 480 (Roche Diagnos-

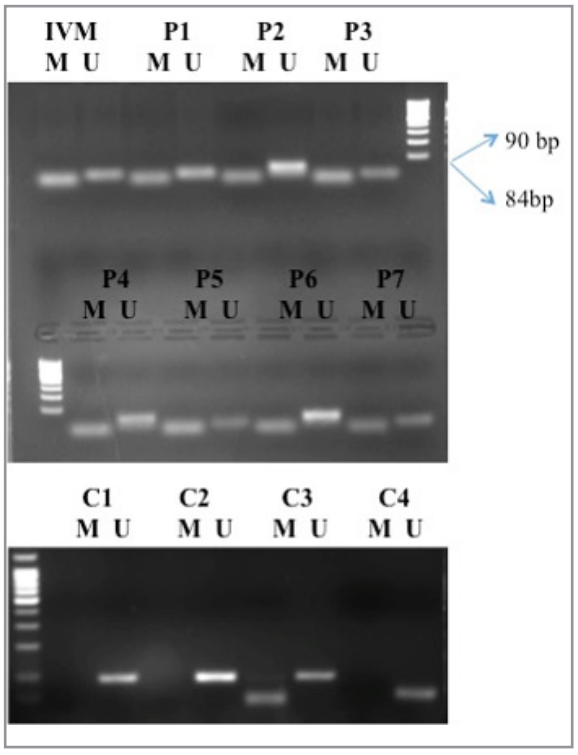

Figure 2. Methylation specific PCR in seven representative acute leukemias patients and four controls on 3% agarose gel electrophoresis stained by EtBr. M; methylated (84bp), U: unmethylated (90bp) P; Patient, C; Contols, IVM; in vitro methylated sample.

tics, Mannheim, Germany), with the LightCyler 480 Fast Start SYBR Green I Master Kit (Roche Diagnostics, Manheim, Germany).

The PCR conditions were prepared according to the instructions of the manufacturer's protocol. 5 pmol of primers and 200 ng of cDNA were used in each run and each sample was studied in duplicate. QRT-PCR primer sequences were; Forward: 5'GAGACAGATGGAGGTGCTGC3'Reverse: 5'GTAAGATGATTGGAATTATCTTCT3'. The specificity of product amplification was confirmed by melting curve analyses and agarose gel electrophoresis. The expression levels of APC were detected by quantitative-real time PCR. The 3 reference genes (β-ACTIN, CYPA and ABL) were studied for normalization as described by Vandesompele et. al.[15]

Table 2. Clinical characteristics and the methylation status of 118 acute leukemia patients

	Methylated n (%)	Unmethylated n (%)
B-ALL (n= 63)	31 (49.2)	32 (50.8)
T-ALL (n= 16)	10 (62.5)	6 (37.5)
AML (n= 39)	25 (64.1)	14 (35.9)
Total (n= 118)	66 (56)	52 (44)

Statistical Analysis

Relative expression values were calculated according to the mathematic model based on the crossing points.[16] Differences between the relative expression levels of cases and controls tested with Mann-Whitney U test. The correlation between methylation status and clinical parameters (sex, age, WBC count at diagnosis etc.) was examined. The Kaplan Mayer method was used to estimate the survival rates. Log rank test was used for survival analyses. P value of 0.05 or less (two-sided) was considered statistically significance. All statistical analyses were done by SPSS 10.0 software.

RESULTS

APC gene promoter methylation is detected in acute leukemia patients. To observe the methylation patterns of the 5' promoter region of the *APC* gene, we analyzed 79 ALL (T and B ALL), 39 AML primary patient samples (Figure 2). In total 56% of acute leukemia patients showed *APC* promoter methylation. Among the acute leukemia patient, 49.2% of B-ALL, 62.5 % of T-ALL and 64.1% of AML samples were hypermethylated (Table 2). Also TALL-1 and FLEB14-4 cell lines were positive for methylation. In contrast, none of our control samples demonstrated *APC* promoter methylation.

APC Expression in Acute Leukemia Patients (AL)

To examine if the promoter methylation effects the expression of the *APC* gene, mRNA levels were also studied in AL patients. All of the acute lymph-

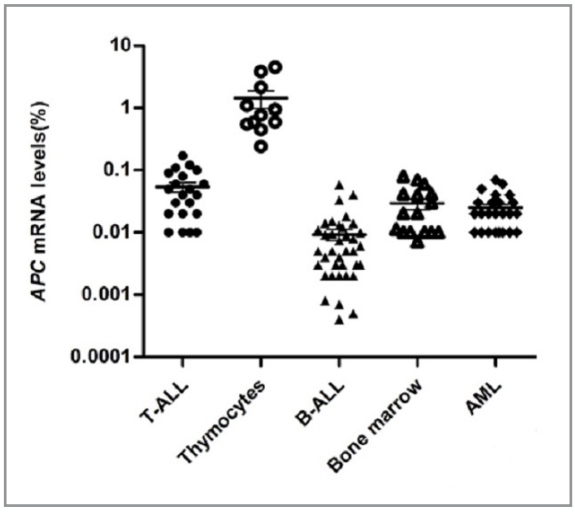

Figure 3. APC mRNA levels in acute leukemia samples and related controls. The APC mRNA levels were normalized by the mean value of 3 house keeping genes (Beta-actin, cyclophilin and ABL)

oblastic leukemia subgroups showed decreased mRNA levels compared to healthy controls. The decrease level in T-ALL and B-ALL patients were statistically significant (p< 0.0001 and p= 0.001, respectively) (Figure 3). Although the AML patients showed lower *APC* expression levels than control bone marrow samples the difference was not statistically significant (p= 0.46) (Figure 3).

There was no correlation between promoter methylation status of *APC* gene and clinical parameters (age, sex, WBC count, translocation status, etc.) of the samples. *APC* gene methylation (p= 0.83) or mRNA expression levels (p= 0.56) did not show a direct effect on overall survival (Figure 4).

DISCUSSION

As a negative regulator, *APC* gene has an important role in WNT pathway. Inactivation of *APC* gene may cause abnormal and constitutive activation of WNT signaling pathway. There are two possible mechanisms in silencing of *APC*, gene mutations or promoter hypermethylation. *APC* gene mutations are highly frequent in colon cancer or hepatocellular carcinoma patients.[17,18] In our previous study, we have examined the mutations in B-CATENIN and its destruction complex members, including *APC* gene, in acute leukemia patients and found that ligand independent activation of WNT due to *APC* mutations is not a common event in acute leukemia like in solid tumors.[13] On the other hand, aberrant methylation in promoter region is known as an alternate mechanism for transcriptional silencing. Recent studies clearly showed epigenetic mechanisms have a prominent role in cancer.[12] The promoter methylation of WNT pathway components was shown in several cancer types.[19-24]

APC promoter methylation was found in gastric cancers and also 48% of the adult T-ALL patients.[23,25] Here we showed that 56% of acute leukemia patients have the promoter methylation of *APC*

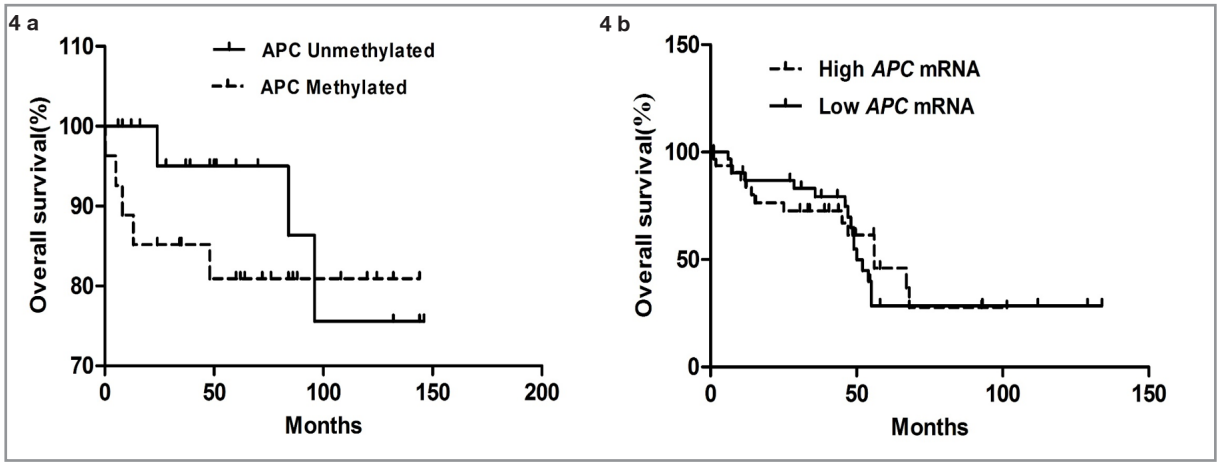

Figure 4. Overall Survival (OS) analysis in acute leukemia patients according to their methylation **(4a)** and expression status **(4b)**

gene and in line with the literature among these around 50% of the T-ALL patients were methylated. On the other hand, Yang et al. declared that they have found no *APC* methylation in B-ALL patients but here we detected *APC* methylation in almost 50% of the pediatric B-ALL patients as well. This discrepancy might be due to difference between the CpG islands region studied. It was previously shown that *APC* gene was highly methylated in breast cancer samples but not in AML patients.[26] In our study the methylation status of AML samples is lower than ALL samples and the expression difference was not significant than control.

Differential methylation of *APC* promoter occurs among patient subgroups and cell lines, but not in healthy individuals, suggesting that hypermethylation of promoter CpG islands of the *APC* gene is a tumor-specific change in acute lymphoblastic leukemia.

In addition to gene deletions or mutations, methylation at CpG sites in the promoter region is an alternative pathway for gene inactivation. QRT-PCR results showed significantly lower *APC* mRNA levels in B and T cell leukemias and mostly in immature group. Our observation implicated that the methylation of the *APC* gene is associated with transcriptional silencing in ALL. The hypermethylation of *APC* gene is the main event that causes the low *APC* mRNA level in acute lymphoblastic leukemia and the epigenetic changes in WNT negative regulators can contribute to the leukemogenesis by untimely activation of WNT pathway.

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